J. Microbiol. Biotechnol. (2003), **13**(4), 628–631

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Purification and Biochemical Characterization of Recombinant Alanine Dehydrogenase from *Thermus caldophilus* GK24

BAE, JUNG-DON[2], YOUN-JEUNG CHO[1], DOO-IL KIM[2], DAE-SIL LEE[2], AND HYUN-JAE SHIN[1]*

[1]*EnzBank, Inc. 309, Bioventure Center (BVC), KRIBB, Yusong, Daejon 305-333, Korea*
[2]*Molecular Glycobiology Research Unit, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Yusong, Daejon 305-333, Korea*

Received: January 3, 2003
Accepted: June 11, 2003

Abstract The recombinant alanine dehydrogenase (ADH) from *E. coli* containing *Thermus caldophilus* ADH was purified to homogeneity from a cell-free extract. The enzyme was purified 38-fold with a yield of 68% from the starting cell-free extract. The purified enzyme gave a single band in polyacrylamide gel electrophoresis, and its molecular weight was estimated to be 45 kDa. The pH optimum was 8.0 for reductive amination of pyruvate and 12.0 for oxidative deamination of L-alanine. The enzyme was stable up to 70°C. The activity of the enzyme was inhibited by 1 mM Zn^{2+}, 20% hexane, and 20% $CHCl_3$. However, 10 mM Mg^{2+} and 40% propanol had no effect on the enzyme activity. The Michaelis constants (K_m) for the substrates were 50 μM for NADH, 0.2 mM for pyruvate, 39.4 mM for NH_4^+, 2.6 mM for L-alanine, and 1.8 mM for NAD^+.

Key words: Alanine dehydrogenase, characterization, enzyme purification, *Thermus caldophilus* GK24

Alanine dehydrogenase (L-alanine: NAD^+ oxidoreductase, EC 1.4.1.1, shortly ADH), catalyzing a reversible oxidative deamination of L-alanine to pyruvate, has an important role in carbon and nitrogen metabolism in various microorganisms by providing a link between carbohydrate and amino acid metabolisms [13]. *Bacillus* ADH apparently plays a part in the catabolism of L-alanine to pyruvate and, in further catabolism by the tricarboxylic acid cycle, both of these catabolic processes provide the energy necessary for sporulation during the differentiation of these cells [3, 13, 19]. When carrying out the amination of pyruvate to L-alanine, the enzyme can be used for nitrogen assimilation, and it is so used in *Rhodobacter capsulatus* and *Streptomyces aureofaciens* [2, 25]. In addition, the ADH involved in anaerobic catabolic processes has been investigated in the *Desulfotomaculum ruminis* and two marine *Desulfovibrio* strains, where the enzyme is involved in the degradation of L-alanine as an energy source [21]. The biochemical properties of the enzyme purified from various bacteria have been characterized [14, 18, 20, 24], and several bacterial ADH genes have been cloned [1, 5, 9, 19]. Ohshima *et al.* and Vali *et al.* reported an abundant occurrence of ADH in several thermophilic strains [14, 17, 24]. The enzymes from thermophiles have more potential usefulness as a technical catalyst with higher stability than those counterparts from the mesophiles [7, 12, 16]. In the present study, ADH from *Thermus caldophilus* GK 24 [6, 23] was purified, and the kinetic properties were investigated.

The ADH gene is composed of 1107 base pairs and encodes a polypeptide of 369 amino acid residues. The plasmid pJR18 for the high expression of the ADH gene (AY293734) was constructed and transformed to *E. coli* MV1184 [10]. Recombined *E. coli* was grown at 37°C and 200 rpm in LB medium containing ampicillin of 100 μg/ml. When cell growth reached 0.5–0.7 O.D. at 600 nm, 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside, Sigma, U.S.A.) was added to the culture medium and then the cells were grown continuously for 7 h. After removing the culture supernatant by centrifugation at 10,000 ×g for 20 min, the resulting cells were disintegrated by sonication, and then the unbroken cells and cell debris were removed by centrifugation at 10,000 ×g for 20 min. The supernatant was used as a starting enzyme source for the purification processes. The crude enzyme preparation was treated by heating at 70°C for 30 min. A chromatographic step procedure was performed at room temperature with a FPLC AKTA system (Pharmacia, Uppsala, Sweden). Two hundreds μl of enzyme preparation were loaded onto a column of Hiprep 16/10 DEAE FF (10×1.6 cm, Pharmacia) pre-equilibrated with 50 mM potassium phosphate buffer (pH 8.0). The proteins were eluted with a linear gradient of

*Corresponding author
Phone: 82-42-864-0057; Fax: 82-42-864-0058;
E-mail: shinhj@enzbank.com

Table 1. Summary of purification of alanine dehydrogenase from *Thermus caldophilus* GK 24.

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	137	273	2	100	1
Heat treatment	51	192	4	70	2
FPLC (DEAE column)	3	185	75	68	38

0-30%, 30-60%, and 60-100% of 1 M NaCl at a flow rate of 5 ml/min. Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay method with bovine serum albumin (BSA) as the standard (Pierce, U.S.A.). Enzyme activity was assayed in 50 mM potassium phosphate buffer (pH 8.0) at 37°C. One ml of the reaction mixture for the oxidative deamination contained 10 μmol of L-alanine, 2 μmol of NAD (Sigma, MO, U.S.A.), 100 μmol of potassium phosphate buffer (pH 8.0), and the enzyme. The reaction was initiated by the addition of enzyme, and at its completion the rate of O.D. increase was measured at 340 nm with an Ultrospec 3100 pro UV/visible spectrophotometer (Pharmacia). The assay system for the reductive amination consisted of 10 μmol of sodium pyruvate, 0.4 mmol of NH_4Cl/NH_4OH (pH 8.0), 0.15 μmol of NADH (Sigma, MO, U.S.A.), and 100 μmol of Tris/HCl buffer (pH 8.0). The reaction rate was determined by measuring the rate of decrease in absorbance at 340 nm.

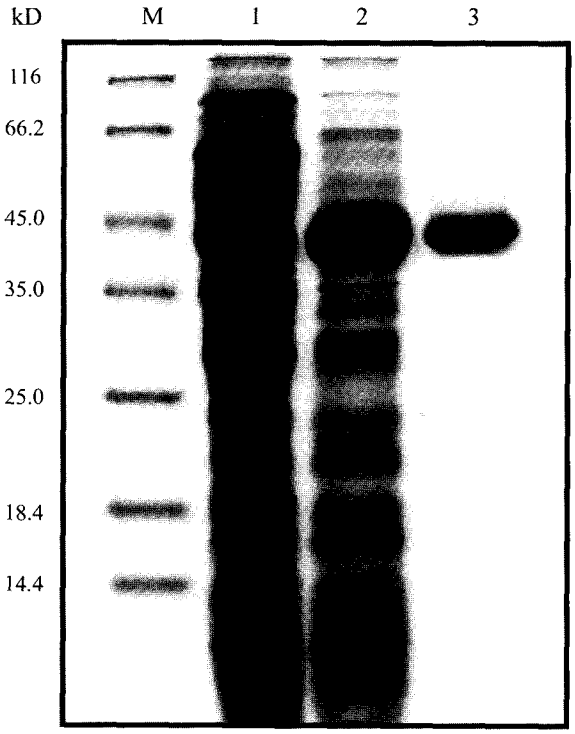

Fig. 1. SDS-PAGE of the purified alanine dehydrogenase.
M: standard marker; lane 1: Crude enzyme; lane 2: After heat treatment; lane 3: After ion exchange chromatography.

$$\text{L-Alanine} + H_2O + NAD^+ \underset{\text{(Reductive amination: } \leftarrow\text{)}}{\overset{\text{(Oxidative deamination: } \rightarrow\text{)}}{\rightleftharpoons}} \text{Pyruvate} + NH_3 + NADH$$

One unit of enzyme is defined as the amount of enzyme to catalyze the formation of 1 μmol of NADH per min in the oxidative deamination. ADH was obtained as an intracellular enzyme [22]. The enzyme was purified about 38-fold simply by a two-step purification process, involving heat treatment and anion exchange column chromatography (Table 1). The enzyme preparation at each purification step was analyzed by SDS-PAGE (Fig. 1). The final enzyme preparation was found to be purified with a yield of 68%, based on the crude enzyme (Table 1). As shown in Fig. 1, SDS-PAGE revealed a single protein band of a molecular mass of 45 kDa, which is the same as the theoretical value for this band. The molecular mass of many other microbial ADHs from *Thermus thermophilus*, *Bilophila wadsworthia*, *Phormidium lapideum*, and *Bacillus sphaericus* were around 48, 42, 41, and 38 kDa, respectively [11, 14, 18, 24]. The optimum pH of the enzyme activity was determined by measuring the activity within the pH range from 4.0 to

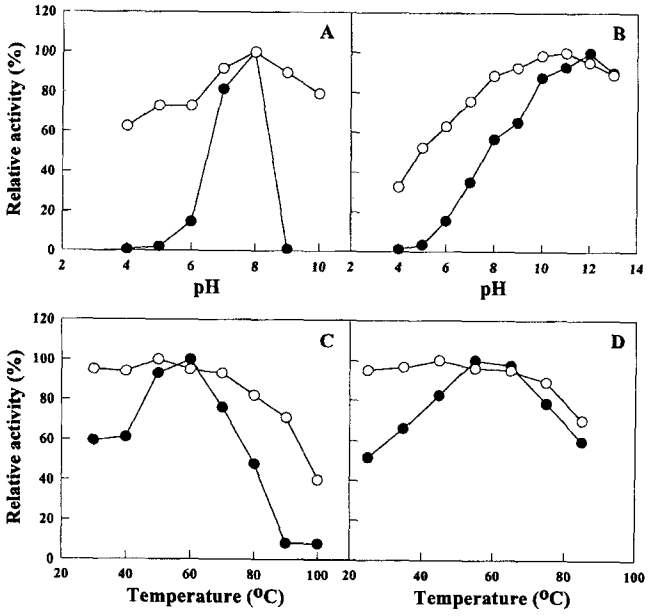

Fig. 2. Effect of pH (●) on the alanine dehydrogenase activity for amination (A) and deamination (B) reactions, and pH stability (○); effect of temperature (●) on the alanine dehydrogenase activity for amination (C) and deamination (D) reactions, and thermal stability (○).

Table 2. The effect of various organic solvents on the activity of alanine dehydrogenase.

Organic solvents	Relative activity (%)	
	Concn. 20% (v/v)	Concn. 40% (v/v)
None	100	100
Ethyl acetate	105	60
Hexane	48	10
Methanol	89	69
Ethanol	96	81
Propanol	122	110
Butanol	114	56
$CHCl_3$	33	12

13.0 (Sodium acetate buffer, 4.0-5.0; Potassium phosphate buffer, 6.0-8.0; CAPS buffer, 9.0-11.0; Borate/NaOH buffer, 12.0-13.0) by incubating the enzyme. As shown in Figs. 2A and 2B, the maximum activity of the enzyme for the oxidative deamination of L-alanine was pH 12.0, and for the reductive amination of pyruvate was pH 8.0. This property was very similar to those of the intact *Thermus thermophilus* enzyme [24]. The pH stability of the ADH was determined by incubating the enzyme for 24 h at 25°C in 50 mM buffer of different pH values, and then measuring the remaining activities. The enzyme was stable in the pH range of 8.0-13.0 and 7.0-9.0 for the deamination and amination reactions, respectively (Figs. 2A and 2B). To examine the effect of temperature on enzyme activity, enzyme reactions were performed at various temperatures ranging from 25 to 100°C. The optimum temperature for ADH activity was approximately 55 to 60°C for the deamination and amination reactions (Figs. 2C and 2D). Thermal stability of the enzyme was estimated by measurement of residual activity after preincubation at various temperatures. The enzymes were stable up to 70°C for the deamination and amination reactions (Figs. 2C and 2D). The effect of various chemicals on the reductive activity of the amination reaction was examined. The activity of the enzyme was strongly inhibited by 1 mM Zn^{2+}, 20% hexane, and 20% $CHCl_3$, however, the enzyme was not influenced by 20% ethylacetate and 20% butanol. Ions such as Mn^{2+} and Ca^{2+} were also

Table 3. The effect of various metal ions and detergents on the activity of alanine dehydrogenase.

Metal ions	Relative activity (%)	
	Concn. 1 mM	Concn. 10 mM
None	100	100
$CaCl_2$	64	12
$MgCl_2$	101	99
$MnCl_2$	44	10
$ZnCl_2$	5	0
SDS	49	10
EDTA	97	68

Table 4. The Michaelis constant of the purified alanine dehydrogenase.

Substrate	Michaelis constant (K_m, mM)
Alanine	2.6
NAD^+	1.8
Pyruvate	0.2
NADH	0.05
NH_4^+	39.4

inhibitory. However, 10 mM Mg^{2+} and 40% propanol did not affect the enzyme activity, and it was slightly inhibited (3%) by 1 mM EDTA (Tables 2 and 3). These results are similar to those of other microbial ADHs. Zn^{2+} was reported to be inhibitory on the activity of ADH from *Bacillus subtilis* [8], and EDTA had no effect on the activity of the enzymes from *Bacillus sphaericus* and *Phormidium lapideum* [14, 18]. The K_m values for the substrates in the forward (oxidative deamination) and reverse reactions (reductive amination) were determined at the optimum pH and at 60°C for each direction, and were obtained from the secondary plots of intercepts rather than the reciprocal concentrations of the other substrate. The K_m values estimated for the oxidative deamination were 2.6 mM for L-alanine and 1.8 mM for NAD^+ and, for the reductive amination, they were 0.2 mM, 0.05 mM, and 39.4 mM for pyruvate, NADH, and ammonia, respectively (Table 4). The ADH of *Thermus* sp. had apparent K_m values for the substrate that were of the same magnitude as those of ADHs from other species. The K_m value of 2.6 mM for L-alanine belongs to the lower K_m values in comparison with other ADHs [6, 15], making the enzyme apparently suitable for use in oxidative deamination. This conclusion is supported by the relatively high K_m value of 39 mM for ammonia. When performing the oxidative deamination of alanine, the enzyme can be used for the estimation of the quantity of alanine in the fermentation broth, since this alanine dehydrogenase has relatively low K_m values for alanine.

REFERENCES

1. Andersen, A. B., P. Andersen, and L. Ljungqvist. 1992. Structure and function of a 40,000-molecular-weight protein antigen of *Mycobacterium tuberculosis*. *Infect. Immun.* **60:** 2317-2323.
2. Caballero, F. J., J. Cardenas, and F. Castillo. 1989. Purification and properties of L-alanine dehydrogenase of the phototrophic bacterium *Rhodobacter capsulatus* E1F1. *J. Bacteriol.* **171:** 3205-3210.
3. Frankel, A. D. and R. F. Jones. 1980. Changes in enzyme activity during differentiation in *Chlamydomonas reinhardtii*. *Biochim. Biophys. Acta* **630:** 157-164.

4. Hoe, H.-S., I. G. Joe, H.-J. Shin, H.-J. Jeon, H.-K. Kim, J. S. Lee, Y.-S. Kim, D.-S. Lee, and S. T. Kwon. 2002. Cloning expression of the gene for inorganic pyrophosphatase of *Thermus caldophilus* GK 24 and properties of the enzyme. *J. Microbiol. Biotechnol.* **12:** 301-305.
5. Hutter, B. and M. Singh. 1998. Host vector system for high-level expression and purification of recombinant, enzymatically active alanine dehydrogenase of *Mycobacterium tuberculosis*. *Gene* **212:** 21-29.
6. Hutter, B. and M. Singh. 1999. Properties of the 40 kDa antigen of *Mycobacterium tuberculosis*, a functional L-alanine dehydrogenase. *Biochem. J.* **343:** 669-672.
7. Johnvesly, B., S. Virupakshi, G. N. Patil, Ramalingam, and G. R. Naik. 2002. Cellulase-free thermostable alkaline xylanase from thermophilic and alkalophilic *Bacillus* sp. JB-99. *J. Microbiol. Biotechnol.* **12:** 153-156.
8. Kim, S. J., Y. J. Kim, M. R. Seo, and B. S. Jhun. 2000. Regulatory mechanism of L-alanine dehydrogenase from *Bacillus subtilis*. *Bull. Korean Chem. Soc.* **21:** 1217-1221.
9. Kuroda, S., K. Tanizawa, Y. Sakamoto, H. Tananka, and K. Soda. 1990. Alanine dehydrogenases from two *Bacillus* species with distinct thermostabilities: Molecular cloning, DNA and protein sequence determination, and structural comparison with other NAD(P)$^+$-dependent dehydrogenases. *Biochemistry* **29:** 1009-1015.
10. Kwon, S. T., J. S. Kim, J. H. Park, H. K. Kim, and D.-S. Lee. 1997. Cloning and analysis of the DNA polymerase-encoding gene from *Thermus caldophilus* GK24. *Mol. Cell* **7:** 264-271.
11. Laue, H. and A. M. Cook. 2000. Purification, properties and primary structure of alanine dehydrogenase involved in taurine metabolism in the anaerobe *Bilophila wadsworthia*. *Arch. Microbiol.* **174:** 162-167.
12. Lee, S. G., H. S. Ro, S. P. Hong, E. H. Kim, and M. H. Sung. 1996. Production of L-DOPA by thermostable tyrosine phenol-lyase of a thermophilic *Symbiobacterium* species overexpressed in recombinant *Escherichia coli*. *J. Microbiol. Biotechnol.* **6:** 98-102.
13. McCowen, S. M. and P. V. Phibbs. 1974. Regulation of alanine dehydrogenase in *Bacillus licheniformis*. *J. Bacteriol.* **118:** 590-597.
14. Ohshima, T., M. Sakane, T. Yamazaki, and K. Soda. 1990. Thermostable alanine dehydrogenase from thermophilic *Bacillus sphaericus* DSM 462. *Eur. J. Biochem.* **199:** 715-720.
15. Ohshima, T. and K. Soda. 1979. Purification and properties of alanine dehydrogenase from *Bacillus sphaericus*. *Eur. J. Biochem.* **100:** 29-30.
16. Ohshima, T. and K. Soda. 1989. Thermostable amino acid dehydrogenase: Applications and gene cloning. *Trend Biotechnol.* **7:** 210-214.
17. Ohshima, T., C. Wandrey, M. Sugiura, and K. Soda. 1985. Screening of thermostable leucine and alanine dehydrogenases in thermophilic *Bacillus* strains. *Biotechnol. Lett.* **7:** 871-876.
18. Sawa, Y., M. Tani, K. Murata, H. Shibata, and H. Ochiai. 1994. Purification and characterization of alanine dehydrogenase from a cyanobacterium, *Phormidium lapideum*. *J. Biochem.* (Tokyo) **116:** 995-1000.
19. Siranosian, K. J., K. Ireton, and A. D. Grossman. 1993. Alanine dehydrogenase (ald) is required for normal sporulation in *Bacillus subtilis*. *J. Bacteriol.* **175:** 6789-6796.
20. Smith, M. T. and S. W. Emerich. 1993. Alanine dehydrogenase from soybean nodule bacteroids: Purification and properties. *Arch. Biochem. Biophys.* **304:** 379-385.
21. Stams, A. and T. Hansen. 1986. Metabolism of L-alanine in *Desulfotomaculum ruminis* and two marine *Desulfovibrio* strains. *Arch. Microbiol.* **145:** 277-279.
22. Suh, C.-I., J.-M. Lim, and H.-C. Sung. 2001. Purification and characterization of extracellular and intracellular glutamine synthetases from *Mycobacterium bovis* BCG. *J. Microbiol. Biotechnol.* **11:** 946-950.
22. Tajuchi, H., H. Matsuzawa, and T. Ohta. 1984. Lactate dehydrogenase from *Thermus caldophilus* GK24, an extremely thermophilic bacterium. *Eur. J. Biochem.* **145:** 283-290.
23. Vali, Z., F. Kilar, S. Lakatos, S. A. Venyaminov, and P. Zavodszky. 1980. L-alanine dehydrogenase from *Thermus thermophilus*. *Biochim. Biophys. Acta* **615:** 34-47.
24. Vancurova, I., A. Vancura, J. Volc, J. Neuzil, M. Flieger, G. Basarova, and V. Behal. 1988. Purification and partial characterization of alanine dehydrogenase from *Streptomyces aureofaciens*. *Arch. Microbiol.* **150:** 438-440.